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(54) **Expression of the recombinnant tumor necrosis factor binding protein I (TBP-I).**

(57) Tumor Necrosis Factor Bindding Protein I (TBP-I), precursors and analogs thereof, are produced by transfecting eukaryotic cells with an expression vector comprising a DNA molecule encoding the whole human type I TNF receptor or a solublole domain thereof, and culturing the transfected cells, whereby the soluble proteins are secreted into the meddium.

EP 0 433 900 A1

EXPRESSION OF THE RECOMBINANT TUMOR NECROSIS FACTOR BINDING PROTEIN I (TBP-I)

The present invention reblates to human Tumor Necrosis Factor (TNF) Binding Protein I, herein designated TBP-I, and more particularly, to the cloning of the gene coding for said protein and its expression in host cells.

TNF-α and TNF-β (lymphootoxin) are structurally related polypeptide cytokines, produced primarily by mononuclear leukocytes, whosse effects on cell function constitute a major factor in the elicitation of the inflammatory response. The TTNFs affect cells in different ways, some of which resemble the functional modes of other inflammatory mmediators, like interleukin-1 (IL-1) and interleukin-6 (IL-6). What appears most distinctive regarding the activiity of the TNFs is that many of their effects can result in cell and tissue destruction. Increasing evidennce that over-induction of these destructive activities contributes to the pathogenesis of a number of ddiseases, makes it of particular interest to elucidate their mechanisms and the ways they are regulated (Old, L.J. (1988) Sci.Am. 258, pp. 41-49).

High affinity receptors, to which both TNF-α and TNF-β bind (Beutler, B.A., et al. (1985) J.Exp.Med. 161, pp. 984-995) play a keyy role in the initiation and in the control of the cellular response to these cytokines. These receptors arre expressed on the surfaces of a variety of different cells. Studies showing that antibodies reacting with ttheir extracellular portions affect cells in a manner very similar to the TNFs, demonstrate that the receptorss and cellular components associated with them are sufficient to provide the intracellular signalling for the ebffects of the TNFs (Espevik, T., et al., (1990) J.Exp.Med. 171, pp. 415-426).

Other studies have shownn that molecules related to the TNF receptors (TNF-Rs) exist also in soluble forms. Two immunologically ddistinct species of such soluble TNF-Rs, designated TNF Binding Proteins I and II, or TBP-I and TBP-II, reespectively, were recently isolated from human urine (Engelmann, H., et al., (1989) J.Biol.Chem. 264, pp. 111974-11980; Engelmann, H., et al., (1990) J.Biol.Chem. 265, pp. 1531-1536; Olsson, I., et al., (1989) Eur.J.HHaematol. 42, pp. 270-275; Seckinger, P., et al., (1989a) J.Biol.Chem. 264, pp. 11966-11973). Immunological evidence indicated that the two proteins are structurally related to two molecular species of the cell surface TNF-R (the type I and type II receptors, respectively). Antibodies to each of the two soluble proteeins were shown to block specifically the binding of TNF to one of the two receptors and could be used tto immunoprecipitate the receptors. Antibodies against one of the two soluble proteins (TBP-I) were also fdound to induce effects characteristic of TNF in cells which express the immunologically cross-reactivee cell receptors (Engelmann, H., et al., (1990) ibid.). Like the cell surface receptors for TNF, the soluble forms of these receptors specifically bind TNF and can thus interfere with its binding to cells. It was suggeested that they function as physiological inhibitors of TNF activity (Engelmann et al., 1989 (ibid.); Olsson et al., 1989 (ibid.); Seckinger et al., 1989a (ibid.)).

Soluble forms of cell surfface receptors may be derived from the cell surface form of the receptor by proteolytic cleavage, or by a ddifferent mechanism proposed in two recent studies describing the cloning of the cDNAs for the receptors to IL-4 and IL-7. Besides cDNA clones encloding the full length receptors, clones which encode truncateod, soluble forms of these receptors were also isolated in these studies. It was suggested that these latter clclones are derived from transcripts specifically encoding soluble forms of the receptors, transcribed from thhe same genes which encode the cell surface forms, but differently spliced (Mosley, B., et al., (1989) Cell 59, pp. 335-348; Goodwin, R.G., et al., (1990) Cell 60, pp. 941-951).

Two recent studies have described the molecular cloning and expression of human type I TNF cell surface receptor (Loetscher, HH., et al. (1990) Cell 61, pp. 351-359; Schall, T.J., et al., (1990) Cell 61, pp. 361-370).

The present invention relaates to the production of human TBP-I, precursors and analogs thereof, by a method comprising transfectioon of eukaryotic, preferably CHO cells, with an expression vector comprising a DNA molecule encoding the wwhole type I human TNF receptor or a soluble domain thereof. When the whole DNA molecule is used, solublele proteins are produced by the transfected cells, along with the cell surface receptor, and are secreted intoo the medium.

The invention further relatites to soluble proteins selected from precursors and analogs of TBP-I, which are secreted into the mediumm by eukaryotic cells transfected with a DNA molecule encoding the whole human type I TNF receptor or a soluble domain thereof.

Figure 1 shows the nucleootide sequence of the type I TNF receptor cDNA and the predicted amino acid sequence of the encoded protdein.

(A) shows the probes used for screening for the cDNA, wherein:

(a) shows the NH_2-terminal amino acid sequence of TBP-I;

(b) shows synthetic oliggonucleotide probes, designed on the basis of the NH_2-terminal amino acid sequence, used for initiabl screening; and

(c) and (d) are probes ooverlapping with (b), used to confirm the validity of clones isolated in the initial screening.

(B) is the schematic presenntation of the cDNA clones isolated from a human colon (C2) and from CEM-lymphocytes (E13) librariess and a diagram of the complete cDNA structure. Untranslated sequences are represented by a line. Codding regions are boxed. The shaded portions represent the sequences which encode the signal peptide aand the transmembrane domains.

(C) shows the hydropathyy profile of the predicted amino acid sequence of the TNF receptor. Hydrophobicity (above the line) and hydrophilicity (below the line) values were determined using the sequence analysis softwaree package of the University of Wisconsin genetic computer group (UWCG) according to Kyte and Dooolittle (1982). The curve is the average of the hydrophobicity index for each residue over a window of niine residues.

(D) depicts the nucleotidee and predicted amino acid sequences of the type I TNF receptor. The presumptive start and stop signals are denoted by asterisks; the three sequences derived from TBP-I by broken overlining; the transmembrane and leader domains by round-ended boxes; and the four repetitive sequences in the extracelluular domain by thick underlining. Cysteine residues are boxed. Glycosylation sites are overlined and the presumptive polyadenylation signal is underlined.

Figure 2 shows the deteoction of type I TNF-R using monoclonal antibodies to TBP-I in CHO cells transfected with E13 cDNA. CHO cells, clones R-18 (transfected with an expression vector in which the E13 cDNA Was placed under the ccontrol of an SV40 promoter) and C-6 (control; a clone of cells transfected with an expression vector in which E13 was placed in the inverse orientation), and HeLa cells, were stained with the anti-TBP-I monoclonal antitibodies 17, 18, 20 and 30 followed by incubation with FITC conjugated anti-mouse F(ab). Fluorescence inttensity was compared with that observed when a mouse monoclonal antibody against TNF was used in the fifirst step of the staining as a control.

Figure 3 shows reversed pphase HPLC of the CHO-produced, soluble form of the type I TNF-R. A concentrate of the condiitioned medium of the CHO R-18 clones (see Fig. 2) and a concentrate of the CHO C-6 clone to which 3 ng pure TBP-I was added, were applied to an Aquapore RP300 column. Elution was performed with a gradieent of acetonitrile in 0.3% aqueous trifluoroacetic acid (---). Fractions were examined for content of proteirin (——) and of the soluble form of the type I receptor by an ELISA

(■),

(as described in Example 1: Procedures). None of the eluted fractions of a concentrate of the CHO C-6 clone, without addition of TBFP-I, was found to contain any detectable amounts of the soluble form of the receptor (not shown).

Figure 4 shows the timne course of the release of COOH-terminal amino acids from TBP-I by carboxypeptidase Y.

Figure 5 shows the consstruction of plasmid pSV-TBP, which contains the DNA sequence encoding TBP-I fused to the strong SV40 early gene promoter.

Figure 6 shows the connstruction of the plasmid pCMV-TBP, which contains the DNA sequence encoding TBP-I fused to the cytomegalovirus (CMV) promoter.

Purified TBP-I isolated froom human urine was described in European Patent Application EP 0 308 378 of the present applicants and shown to contain at the N-terminus the amino acid sequence shown in Fig. 1Aa.

The COOH-terminal of TB3P-I was determined now and shown to contain a major fraction containing the sequence Ile-Glu-Asn denotedd by broken overlining at positions 178-180 in Fig. 1D, and at least one minor fraction including at least two further amino acids Val-Lys at positions 181-182.

The invention relates to aa method for the production of a soluble recombinant protein selected from human Tumor Necrosis Factoor Binding Protein I (TBP-I), biologically active precursors and analogs thereof, which comprises:

i) transfecting eukaryotic ceells with an expression vector comprising a DNA molecule encoding the whole type I human TNF receptorr or a soluble domain thereof, and

ii) culturing the transfected cells, whereby the desired protein is produced and secreted into the medium.

The DNA sequence encooding the whole type I TNF receptor is depicted in Figure 1D. The soluble domain thereof includes the sequence down to position 180 (Asn) or 182 (Lys) or even some additional amino acids after position 1822.

The soluble proteins prodduced by the transfected cells according to the method of the invention and

secreted into the medium may have at the N-terminus the sequence Asp-Ser-Val denoted by broken overlining at positions 20-23 in Fig. 1D (TBP-I), or the sequence Leu-Val-Pro at positions 9-11 or Ile-Tyr-Pro at positions 1-3 or any other sequence between Ile(+1) and Asp(20). The proteins may have at the COOH terminal any of the sequences described above. All these soluble proteins, if biologically active with TBP-I-like activity, are encompassed by the invention as precursors and analogs of TBP-I.

According to the invention, oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBP-I, were synthesized by known methods and used for screening for the cDNA coding for TBP-I in cDNA libraries. In a human colon cDNA library, a C2 cDNA insert was found which hybridized to said probes and it was used for further screening in a human CEM-lymphocytes lambda ZAP cDNA library, thus leading to the cDNA shown in Fig. 1D.

The DNAs of positive clones were then inserted into appropriately constructed expression vectors by techniques well known in the art. In order to be capable of expressing a desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding for the desired protein in such a way as to permit gene expression and production of the protein. The gene must be preceded by a promoter in order to be transcribed. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

The DNA molecule comprising the nucleotide sequence coding for a protein comprising the amino acid sequence of TBP-I, i.e. TBP-I, a precursor or an analog thereof, preceded by a nucleotide sequence of a signal peptide and the operably linked transcriptional and translational regulatory signals is inserted into a vector which is capable of integrating the desired gene sequences into the host cell chromosome. The cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector.

In a preferred embodiment, the introduced DNA molecule will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Factors of importance in selecting a particular plasmid or viral vector include the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate precipitation, direct microinjection, etc.

Host cells to be used in this invention may be either prokaryotic or eukaryotic. Prokaryotic hosts, such as bacteria, e.g. E.coli, are used only when the cDNA encoding the soluble domain of the type I TNF receptor is used to transfect the cells. Under such conditions, the protein will not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

Eukaryotic cells are transfected according to the invention with plasmids comprising the cDNA encoding the whole type I TNF receptor. Preferred eukaryotic hosts are mammalian cells, e.g., human, monkey, mouse and chinese hamster ovary (CHO) cells. They provide the soluble form of the protein, besides the cell surface receptor, and provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. The eukaryotic cells may also be transfected with a plasmid comprising a cDNA encoding a soluble domain of the human type I TNF receptor molecule. Preferred mammalian cells according to the invention are CHO cells.

After the introduction of the vector, the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired soluble protein, that is secreted into the medium, and may then be isolated and purified by any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like.

In a preferred embodiment, CHO cells are transfected with the type I TNF-R cDNA shown in Fig. 1D and they produce both the cell surface receptor and TBP-I, its soluble form, and/or precursors and analogs thereof.

The data presented in the present application are consistent with the notion that TBP-I - the soluble form for the type I TNF-R - constitutes a fragment of the cell surface form of this receptor, corresponding to its extracellular domain. The receptor is recognized by several monoclonal antibodies to TBP-I which interact with several spatially distinct epitopes in this protein. The amino acid sequence in the extracellular domain matches the sequence of TBP-I.

Particularly informative with regard to the mechanism of formation of TBP-I is the finding described in the present application, that a soluble form of the type I TNF-R is produced by CHO cells which were transfected with the TNF-R cDNA. This implies that cells possess some mechanism(s) which allow(s) the

formation of the soluble form of the TNF-R from that same transcript that encodes the cell surface form.

The low rate of production of the soluble form of the type I TNF-R by the E13-transfected CHO cells does not necessarily reflect maximal activity. In HT29 cells, the spontaneous release of a soluble form of type I TNF-R occurs at about a 10-fold higher rate than that observed with the CHO-R-18 clone.

A likely mechanism whereby soluble forms of TNF receptors can be derived from the same transcripts which encode the cell surface forms is proteolytic cleavage. Indeed, flanking the amino acid residue which corresponds to the NH_2-terminus of TBP-I there are, within the sequence of amino acids of the receptor, two basic amino acid residues (Lys-Arg) which can serve as a site of cleavage by trypsin-like proteases. The identity of the proteases which might cause cleavage to take place at the COOH terminus of TBP-I is not known.

The invention will be illustrated by the following examples:

EXAMPLE 1: PROCEDURES

A) Determination of amino acid sequences within the TNF-binding proteins TBP-I and TBP-II

The TNF Binding Proteins TBP-I and TBP-II were isolated from concentrated preparations of urinary proteins, as described previously (Engelmann, H., et al., (1990) J.Biol.Chem. 265, pp. 1531-1536) by ligand (TNF) affinity chromatography followed by reversed phase HPLC. TBP-I was cleaved with cyanogen bromide, yielding two peptides which, following reduction and alkylation, were isolated by reversed phase HPLC. The two peptides (CNBr-1 and CNBr-2 in Table I) were subjected to NH_2-terminal sequence analysis or a pulsed liquid gas phase protein microsequencer (Model 475A, Applied Biosystems Inc., Foster City CA). The sequence found for one of the peptides (CNBr-1) was identical to the NH_2 sequence of the intact TBP-I protein.

The COOH terminal sequence of amino acids in TBP-I was determined by digestion of the protein with carboxypeptidase Y followed by sequential analysis of the released amino acids. A sample of pure TBP-I (32μg) was mixed with 1 nmole of norleucine, as an internal standard, dried thoroughly and resuspended in 8 μl 0.1 M sodium acetate buffer, pH 5.5, containing 0.8 μg carboxypeptidase Y (Sigma, St. Louis, MO). Digestion was performed at room temperature. 2 μl Aliquots withdrawn at various time points were acidified by adding 3 μl of 10% acetic acid to each, followed by addition of 15 μl 0.5% EDTA. They were then subjected to automated amino acid analysis (Applied Biosystems, U.K. model 420A). The results (shown in Fig. 4) indicate the sequence -Ile-Glu-Asn-COOH. Minor fractions were detected containing two or more additional amino acids.

Sequences within TBP-II were determined by generation of tryptic peptides of the protein. A sample of pure TBP-II (200 μg) was reduced, alkylated and repurified on an Aquapore RP-300 reversed-phase HPLC column. Fractions containing the modified protein were pooled and the pH was adjusted to 8.0 with $NaHCO_0$. Digestion with TPCK-trypsin (238 U/mg, Millipore Corp., Freehold, NJ) was performed for 16 h. at room temperature at an enzyme to substrate ratio of 1:20 (w/w). The digest was loaded on a C_{10} RP-P reversed phase HPLC column (Synchrom, Linden, IN) and the peptides separated by a linear 0 to 40% acetonitrile gradient in 0.3% aqueous trifluoroacetic acid. The NH_2 terminal amino acid sequences of the peptides and of the intact protein (N-terminus) are presented in Table I. The peptides were numbered according to their sequences of elution from the RP-P column. In the fractions denoted as 39,44,46,53 and 54, where heterogeneity of sequences was observed, both the major and the secondary sequences are presented.

b) Isolation of cDNA clones

Three mixtures of synthetic oligonucleotide probes (Figs. 1Ab, 1Ac) generated from the nucleotide sequence deduced from the NH_2-terminal amino acid sequence of TBP-I (Fig. 1Aa) were used for the screening of cDNA libraries. Initial screenings were carried out with 48-fold degenerated, 26-mers into which deoxyinosine was introduced, wherever the codon ambiguity allowed for all four nucleotides (Fig. 1Ab). The validity of positive clones was examined by testing their hybridization to two mixed 17-mer nucleotide sequences containing 96 and 128 degeneracies, corresponding to two overlapping amino acid sequences which constitute part of the sequences to which the 26-base probes correspond (Fig. 1Ac and d). An oligonucleotide probe corresponding to a sequence located close to the 5' terminus of the longest of the partial cDNA clones isolated with the degenerated probes (nucleotides 478-458 in Fig. 1D) was applied for further screening cDNA libraries for a full length cDNA clone. ^{32}P-labeling of the probes, using T4 polynucleotide kinase, plating of the phages in lawns of bacteria, their screening with the radio-labelled

probes, isolation of the positivee clones and subcloning of their cDNA inserts were carried out using standard procedures (Sambrook, J., et t al., (1989) Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press).

c) Nucleotide sequencing of thne cDNA clones

cDNA inserts isolated froom positive lambda GT11 recombinant phages were subcloned into the pBluescript KS(-) vector. Inseerts found in lambda ZAP phages were rescued by excising the plasmid pBluescript SK(-) in them, usinng the R408 helper phage (Short, J.M., et al., (1988) Nucl.Acids Res. 16, pp. 7583-7600). DNA sequencingg in both directions was done by the dideoxy chain termination method. Overlapping deletion clones ofhf the cDNAs were generated, in both orientations, by digestion of the cDNA with exonuclease III ("Erase a base" kit, Promega Biotec, Madison, WI). Single stranded templates derived from these clones using the RR408 phage were sequenced with a T7 DNA polymerase sequencing system (Promega).

d) Constitutive expression of thhe type I human TNF-R in CHO cells

The E13 insert was introdduced into a modified version of the pSVL expression vector. This construct was transfected, together witith the pSV2-DHFR plasmid which contains the DHFR cDNA, into DHFR deficient CHO cells, using thee calcium phosphate precipitation method. Transfection with a recombinant pSVL vector which contained tthe E13 insert in the inverse orientation served as a control. Cells expressing the DHFR gene were selectedd by growth in nucleotide-free MEM alpha medium containing fetal calf serum which had been dialyzed agaainst phosphate buffered saline. Individual clones were picked out and then further selected for amplificatiion of the transfected cDNAs by growth in the presence of 500 nM sodium methotrexate.

e) Detection of surface-expresssed type I TNF-R in the CHO cells

Binding of radiolabelled huuman rTNF to cells (seeded in 15 mm tissue culture plates at a density of 2.5 X 10^5 cells/plate) was quantittated as described before (Holtmann, H. and Wallach, D. (1987) J.Immunol. 139, pp. 1161-1167).

To examine the binding ofbf monoclonal antibodies against TBP-I to CHO cells, the cells were detached by incubation in phosphate buuffered saline (PBS: 140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 m KCl, 0.5 m $MgCl_2$, 0.9 m $CaCCl_2$), containing 5 mM EDTA and then incubated for 45 min. at 0°C with 50 μg/ml of the test monoclonal aantibody in PBS containing 0.5% bovine serum albumin, and 15 mM sodium azide (PBS/BSA). After washinng the cells with PBS/BSA they were incubated further for 30 min. at 0°C with FITC labelled, affinity purified goat antibody to the F(ab) fragment of mouse IgG (1:20 in PBS/BSA) (Bio-Makor, Israel) and then analyzeed by determining the intensity of fluorescence in samples of 10^4 cells using the Becton Dickinson fluorescecence activated cell sorter 440. Three monoclonal antibodies to TBP-I, clones 17,18 and 20, shown by crosss competition analysis to recognize four spatially distinct epitopes in the TBP-I molecule (European Patent Application No. 90115105.0) and, as a control, a monoclonal antibody against TNF-α (all purified from ascititic fluids by ammonium sulphate precipitation and of the IgG2 isotype), were used.

f) Quantitation of the soluble foorm of the type I TNF-R by ELISA

A sensitive enzyme linkeed immunosorbent assay was set up using TBP-I-specific monoclonal and polyclonal antibodies in a sanodwich technique. Immunoglobulins of the anti-TBP-I mAb clone 20 (European Patent Application No. 901151105.0) were adsorbed to 96-well ELISA plates (maxisorp, Nunc, Denmark) by incubation of the plates for 2 hh. at 37°C with a solution of 25 μg/ml of the antibody in PBS. After incubating the wells further for 2 h. at 337°C with a solution containing phosphate buffered saline, 1% BSA, 0.02% NaN_3 and 0.05% Tween 20) (blocking solution) to block nonspecific further binding of protein, tested samples were applied in aliquuots of 50 μl/well. The plates were then incubated for 2 h. at 37°C, rinsed 3 times with PBS supplementeod with 0.05% Tween 20 (washing solution) and rabbit polyclonal antiserum against TBP-I, diluted 1:500 inn blocking solution, was added to the walls. After further incubation for 12 h. at 4°C the plates were rinsed aggain and incubated for 2 h. with horse raddish peroxidase-conjugated purified goat anti-rabbit IgG. The assayy was developed using 2,2'-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) as a substrate (Sigma). The enzyymatic product was determined colorimetrically at 600 nm. Pure TBP-I served

as a standard.

g) Detection of a soluble form of the type I TNF-R in the growth medium of the transfected CHO cells and its analysis by reversed phase HPLC

The amounts of the soluble form of the type I TNF-R in samples of the medium of the tested CHO cells, collected 48 h after medium replacement, were determined by the immunoassay described above. For analysis of the soluble receptor by reversed phase HPLC the CHO cells were cultured for 48 h. in serum-free medium (nucleotide-free MEM α). The medium samples were concentrated 100-fold by ultrafiltration on an Amicon PM5 membrane and 100 μl aliquots were then applied to an Aquapore RP300 column (4.5 X 30 mm, Brownlee Labs) preequilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with this solution at a flow rate of 0.5 ml/min until all unbound proteins were removed, and then eluted with a gradient of acetonitrile concentration in 0.3% aqueous trifluoroacetic acid, as described before (Engelmann, H., et al., (1989) J.Biol.Chem. 264, pp. 11974-11980). Fractions of 0.5 ml were collected and, after concentration in vacuo, were neutralized with 1 M HEPES buffer pH 9.0. Amounts of soluble type I TNF-R in the fractions were determined by ELISA and the concentration of protein by the fluorescamine method.

EXAMPLE 2

a) Cloning of the cDNA for the Type I TNF-R

To clone the cDNAs which code for the TNF-binding protein, TBP-I, and its related TNF receptor, several cDNA libraries were screened, using 3 overlapping oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBP-I (Fig. 1A). In a lambda GT11 library derived from the mRNA of human colon (randomly primed, Clontech, Palo Alto, CA), four recombinant phages which hybridized with the three probes were detected. The inserts in these four phages were similar in size, and were found to overlap by restriction mapping and sequence analysis.

Complete analysis of the sequence of the longest of the four (C2 in Fig. 1B, deposited on 6.12.1989 with the Collection Nationale de Cultures de Microorganismes (C.N.C.M.), Paris, France, Accession No. I-917) revealed an open reading frame, extended over its entire length. A polypeptide chain encoded in this reading frame fully matches the NH_2-terminal amino acid sequence of TBP-I. Neither an initiation nor a stop codon was found in the C2 insert. Rescreening the colon cDNA library, using another probe corresponding to a sequence found in C2 (see Example 1: Procedures), yielded several other recombinant phages containing inserts that overlap with the C2 insert. However, none of them provided further sequence information on the cDNA in the 5' or the 3' direction. In a lambda ZAP cDNA library derived from the mRNA of CEM lymphocytes (Oligo dT and randomly primed, Clontech) five phages hybridizing with this probe were detected, which contained significantly longer inserts than C2.

The longest insert (E13, Fig. 1B) was sequenced in its entirety (Fig. 1D) and was found to contain the C2 sequence (nucleotides 346-1277 in Fig. 1D) within one long open reading frame of 1365 bp, flanked by untranslated regions of 255 and 555 nucleotides at its 5' and 3' ends, respectively. The potential ATG initiation site, occurring at positions 256-258 in the nucleotide sequence (denoted by an asterisk in Fig. 1D) is preceded by an upstream in-frame termination codon at bases 244-246. The start location is in comformity with one of the possible alternatives for the translation initiation consensus sequence (GGCATGG, nucleotides 253-259).

There is no characteristic poly(A) addition signal near the 3' end of the cDNA. The sequence ACTAAA, at nucleotides 2045-2050, may serve as an alternative to this signal, but with low efficiency. At nucleotides 1965-2000, there are six consecutive repeats of the sequence G(T)n (n varying between 4 and 8).

The size of the protein encoded by the cDNA (about 50 kD) is significantly larger than that of TBP-I. A hydropathy index computation of the deduced amino acid sequence of the protein (Fig. 1C) revealed two major hydrophobic regions (see round-ended boxes in Fig. 1D). One, at its NH_2-terminus, is apparently the signal peptide whose most likely cleavage site lies between the glycine and isoleucine residues designated in Fig. 1D as -1 and +1 respectively. The other major hydrophobic domain, located between residues 191 and 213, is flanked at both ends by several charged amino acids, characteristic of a membrane anchoring domain. As in several other transmembrane proteins, the amino acids confining the hydrophobic domain at its COOH-terminal are basic. The transmembrane domain bisects the predicted protein into almost equally sized extracellular and intracellular domains.

The extracellular domain has 3 putative sites for asparagine-linked glycosylation (overlined in Fig. 1D).

Assuming that the amount of oligosaccharides in the extracellular domain is similar to that reported as present in TBP-I (Seckinger, P., et al., (1989b) Cytokine I, 149 (an abstract)), the molecular size of the mature protein is very similar to that estimated for the type I receptor (about 58kD) (Hohmann, H.P., et al., (1989) J.Biol.Chem. 264, pp. 14927-14934).

b) Features of the predicted amino acid sequence in the Type I TNF-R and relationship to the structure of TBP-I and TBP-II

The amino acid sequence of the extracellular domain of the protein encoded by the E13 cDNA fully matches several sequences of amino acids determined in TBP-I (Table I). It contains the NH_2-terminal amino acid sequence of TBP-I at amino acids 20-32 (compare Fig. 1D and Fig. 1Aa), a sequence corresponding to the COOH terminus of TBP-I at amino acid 178-180; and, also, adjacent to the first methionine located further downstream in the encoded protein, a sequence which is identical to the NH_2-terminal amino acid sequence of a cyanogenbromide cleavage fragment of TBP-I (broken lines in Fig. 1D). There is also a marked similarity in amino acid composition between the extracellular domain of the receptor and TBP-I (Table II).

The most salient feature of this amino acid composition is a very high content of cystein residues (shown boxed in Fig. 1D). The positioning of the cystein residues as well as of other amino acids within the extracellular domain displays a four-fold repetition pattern (underlined in Fig. 1D). The amino acid sequence within the extracellular domain of the TNF-R, which corresponds to the COOH terminal sequence of TBP-I (see Table I and Fig. 4), is located at the COOH terminus of the cystein-rich repeat region. The sequence corresponding to the NH_2 terminal sequence of TBP-I corresponds to a sequence located a few amino acids upstream of the NH_2 terminal end of this region (broken lines in Fig. 1D) in the extracellular domain.

In contrast to the identity of amino acid sequences between TBP-I and the extracellular domain of the type I TNF receptor, sequences examined in the soluble form of the type II TNF-R (TBP-II, Table I) were not identical to any sequence in the type I TNF-R. This finding is expected, considering the lack of immunological crossreactivity between the two receptors (Engelmann, H., et al., (1990) J.Biol.Chem. 265, pp. 1531-1536).

In contrast to the very high content of cystein residues in the putative extracellular domain of the type I TNF-R, there are only 5 cystein residues in the intracellular domain. Between the two which are proximal to the transmembrane domain (positions 227 and 283), extends a stretch of 55 amino acids which is rich in proline residues (15% of the residues) and even richer in serine and threonine residues (36%), most located very close to or adjacent to each other.

EXAMPLE 3

Expression of the type I TNF-R cDNA

To explore the relation between the protein encoded by the E13 cDNA and TBP-I further, this protein was expressed in CHO cells. The E13 cDNA was introduced into an expression vector and was cotransfected with a recombinant vector containing the dihydrofolate reductase (DHFR) cDNA into DHFR-deficient cells. After selection by growth in a nucleotide-free medium, individual clones were amplified by growth in the presence of methotrexate. A number of clones which react with several monoclonal antibodies that bind to spatially distinct epitopes in TBP-I were detected (Fig.2). Expression of the protein was correlated with an increase in specific binding of human TNF to the cells (Table III).

Applying a sensitive immunoassay for TBP-I in which polyclonal antibodies and a monoclonal antibody against this protein were employed, (Procedures, Example 1f) in the medium of CHO cells which express the human TNF-R on their surface, also a soluble form of the protein could be detected (Table III). All of five different CHO clones which expressed the TNF-R, produced this soluble protein. Several other transfected clones which did not express the cell surface receptor did not produce its soluble form either. When analyzed by reversed phase HPLC, the CHO-produced soluble TNF-R eluted as a single peak, with a retention time identical to that of TBP-I (Fig. 3).

EXAMPLE 4

Cloning of the cDNA encoding TBP-I and expression of TBP-I in Chinese Hamster Ovary (CHO) cells

In order to obtain plasmids suitable for efficient expression of the DNA encoding a soluble domain of

the type I TNF receptor in mammalian cells, the gene from position 256 to position 858 of the DNA sequence shown in Fig. 1D, was cloned in two expression vectors: in one plasmid, gene expression was under the SV40 early gene promoter; in the second plasmid, gene expression was under the regulation of the cytomegalovirus (CMV) promoter. These vectors were introduced into CHO cells by $CaPO_4$ coprecipitation with a plasmid DHFR selectable genetic marker.

Construction of Expression Vectors

1) SV40 Early Promoter-TBP-I fusion: Plasmid pSV-TBP.

Constitutive expression of TBP-I can be achieved by using an expression vector that contains the DNA sequence coding for TBP-I fused to the strong SV40 early gene promoter (Fig 5).

Step 1: A DNA fragment coding for TBP-I, Including its signal peptide and extending to amino acid 180 was prepared by PCR amplification. For amplification two oligonucleotides were used as primers: the 5' end primer contains the sequence coding for the first seven amino acids of the signal peptide, preceded by six nucleotides; the 3' end oligonucleotide contains the sequence coding for amino acid residues 174 through 180 followed by two stop codons (TGA and TAA).).

The conditions for PCR amplification are the following:

	Temperature °C	Time min
1 cycle	94 50 72	6 2 4
30 cycles	94 50 72	1 2 4
1 cycle	94 50 72	1 2 12

Step 2: After sequence verification, the amplified DNA fragment was cloned into the HincII restriction site of plasmid pGEM-1 by blunt end ligation. Plasmids pTBP-16 and pTBP-17 are the two plasmids obtained in this way and they differ in the orientation of the TBP-I insert with respect to the cloning vector.

Step 3: The DNA fragment containing TBP-I was excised from plasmid pTBP-17 using the two adjacent restriction sites HindIII (at the 5' end) and BamHI (at the 3' end).

Step 4: Finally, this fragment was cloned between the HindIII and the BclI restriction sites of the expression vector pSVE3.

The resulting plasmid is called pSV-TBP (Fig. 5).

2) CMV promoter-TBP-I fusion: plasmid pCMV-TBP.

Alternatively, constitutive expression of TBP-I can be achieved by using an expression vector that contains the DNA sequence coding for TBP-I fused to the CMV promoter (Fig 6).

The first two steps for the construction of the CMV based vector are identical to the ones described for the construction of the SV40-TBPI fusion plasmid, as described above.

Step 3: The DNA fragment containing TBP-I was excised from plasmid pTBP-17 using the two adjacent restriction sites HindIII (at the 5' end) and XbaI (at the 3' end).

Step 4: Finally, this fragment was cloned between the HindIII and the XbaI restriction sites of the expression vector Rc/CMV.

The resulting plasmid is called pCMV-TBP.

Expression of Human TBP-I in CHO Cells

CHO cells CHO-K1 DHFR⁻, lacking DHFR activity, were transformed by $CaPO_4$ coprecipitation with a 12:1 mixture of uncut pSV-TBP DNA (73 μg) and mpSV2DHFR (6μg) DNA, the latter being the selectable marker. Alternatively, CHO cells were transformed with a 5:1 mixture of pCMV-TBP (30 μg) and mpSV2DHFR (6 μg).

Cells were grown in nutrient mixture F12 (Gibco) with 10% fetal calf serum (FCS) at 37° C in 5% CO_2. For DNA transfection, $5x10^5$ cells were cultured for one day in 9 cm plates. A $CaPO_4$-DNA coprecipitate was prepared by mixing plasmid DNAs, dissolved in 0.45 ml of Tris-HCl pH 7.9, 0.1 mM EDTA with 0.05 ml of 2.5 M $CaCl_2$; therafter, 0.5 ml of 280 mM Na_2PO_4, 50 mM Hepes buffer pH 7.1 was added with gentle mixing. The mixture was kept for 30-40 minutes at room temperature in order to form the precipitate. After adding the $CaPO_4$-DNA to the cells and leaving the cells at room temperature for 30 min, 9 ml of nutrient mixture F12, 10% FCS were added and the cultures returned to the CO_2 incubator for 4 hours. Medium was removed and the cells were osmotically shocked with 10% glycerol in F12 for 4 min. After 48 hours of growth in non-selective medium, the cells were then trypsinized and subcultured 1:10 into selective medium, composed of Dulbecco's modified Eagle's medium (DMEM) (H21, Gibco), 150 μg/ml proline, and 10% FCS which had been extensively dialyzed against phosphate-buffered saline (PBS). In some cases, MEM alpha medium without nucleotides (F20, Gibco) was used. The cultures were kept at 37° C and 10% CO_2 and the medium was changed every 3-4 days. Clones were isolated after about 15 days, trypsinized, and grown to mass cultures.

Transformants able to grow in medium lacking thymidine (DMEM with dialyzed serum) were obtained. Culture supernatants of individual transformant clones or culture supernatant of mixed populations were screened for human TBP-I by measuring the level of secreted protein by the enzyme linked immunoassay described in Example 1f. TBP-I levels of up to 10 ng/ml were detected in culture supernatants of mixed cells populations.

This example shown that TBP-I or a similar soluble protein can be obtained also by transfection of mammalian cells with a DNA encoding the soluble domain of the type I TNF receptor.

EXAMPLE 5

Expression of TBP-I in E. coli.

For expression of TBP-I in E.coli, the sequence coding for the signal peptide and for the first 19 aminoacids (Arg) must be removed (Figure 1D). Moreover, the Asp residue must be preceded by a Met residue. The desired fragment is then cloned into the expression vector pKK223-3 that contains the hybrid tryp-lac promoter. To achieve this goal plasmid pTBP-16 (Fig 5) is cut with the two unique restriction sites StyI and HindIII. StyI restriction site is C/CAAGG and, therefore, it cuts after Pro24. HindIII restriction site is located in the polylinker of the plasmid and downstream from the two added stop codons that follow Asn180 (Fig. 5).

The resulting DNA fragment, coding for TBP-I, has an intact 3' end and a truncated 5' end, where the sequence preceding the StyI site and coding for Asp-Ser-Val-Cys-Pro has been removed.

For cloning of the StyI-HindIII fragment into the expression vector pKK223-3, the following couple of synthetic oligonucleotides are used:

```
             Met Asp Ser Val Cys Pro
5'   AATTC ATG GAT AGT GTG TGT CCC          3'
3'         G TAC CTA TCA CAC ACA GGG GTT C   5'

     EcoRI                          StyI
```

One end of this double s stranded oligonucleotide is an EcoRI restriction site. This end is ligated to the EcoRI site of plasmid pKK2223-3, located downstream to the tryp-lac promoter. The second end of the double stranded oligonucleotitide is a StyI restriction site to be ligated to the StyI of the TBP-I DNA fragment.

The remainder of the seqquence is such that the codons coding for the first five amino acids are restored and that on additional Met coodon is added in front of Asp20. The expression vector is obtained by ligation of plasmid pKK223-3, digested I with EcoRI and HindIII, to the double-stranded synthetic oligonucleotide and to the StyI-HindIII TBPI fragmennt.

E.coli cells are transfecteed with this expression vector in order to produce TBP-I.

Table I: Amino acid sequences of TBP I and TBP II

TBP I:

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
CNBr-1 (=N-terminus)	NH2	Asp	Ser	Val	Cys	Pro	Gln	Gly	Lys	Tyr	ILe	His	Pro	Gln	---		
CNBr-2	NH2	Gly	Gln	Val	Glu	ILe	Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	---
C-terminus	---	ILe	Glu	Asn	COOH												

TBP II:

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
N-terminus	NH2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg	Leu	Arg	Glu	Tyr	Tyr
TRP 35	NH2	Leu	Cys	Ala	Pro	Leu	Arg	Lys															
TRP 39/1	NH2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg													
TRP 39/2	NH2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---										
TRP 44/1	NH2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	Ser	---									
TRP 44/2	NH2	Ser	Cys	Gly	Pro	Ser	Tyr	Pro	Asp	---													
TRP 46/1	NH2	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg									
TRP 46/2	NH2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---								
TRP 50	NH2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---								
TRP 54/1	NH2	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	Lys													
TRP 54/2	NH2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg					
TRP 53/1	NH2	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg							
TRP 53/2	NH2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg													
TRP 60	NH2	Ile	Cys	Thr	Cys	Arg	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	---								
TRP 62	NH2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	Lys		
TRP 65	NH2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	Lys		
TRP 67	NH2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys		
TRP 84	NH2	Thr	Ser	Asp	Thr	Val	Cys	Asp	Ser	Cys	Glu	Asp	Ser	Thr	Tyr	Thr	Gln	Leu	Trp	---			

Table II. *Similarity of the amino acid compositions of the TNF binding protein TBPI and a corresponding region in the extracellular domain of the TNF-R (type I)*

Amino acid	mol/100 mol of amino acids	
	TBPI*	Residues 20-180 in the extracellular domain**
Ala 1.7	1.2	
Cys	12.8	14.9
Asp + Asn	10.9	11.1
Glu + Gln	13.9	12.4
Phe	3.2	3.1
Gly	6.3	5.6
His	4.4	4.3
Ile	2.8	2.5
Lys	6.2	6.2
Leu	8.0	6.8
Met	0.4	0.6
Pro	3.8	3.1
Arg	4.7	4.3
Ser	8.1	9.3
Thr	6.1	6.2
Val	4.2	4.3
Trp	-	0.6
Tyr	2.4	3.1

* According to Olsson et al., 1989

**Residue 20 corresponds to the NH_2-terminal amino acid of TBPI. Residue 180 is the COOH-terminal residue of TBPI.

Table III. *Expression of thhe cell surface and soluble forms of human type I TNF-R in CHO cells*

CHO cell clone	Specific binding of TNF (CPM/10^6 cells)	cells expressing human cell surface TNF-R (% fluorescent cells)	human soluble type I TNF receptors (pg/ml)
nontransfected	180±45	<1%	<0.03
C6	175±50	<1%	<0.03
R-16	550±60	73%	30
R-18	610±40	80%	40

The R-16 and R-18 clones coonsist of cells transfected with a recombinant expression vector containing E13 cDNA. C-6 cells weree transfected with a control vector (see Fig. 3). Binding of radiolabelled TNF to the cells was determined in pentaplicate samples. Detection of immunoreactive receptors on the surface of the cells wwas carried out using combined 17, 18, 20 and 30 anti TBPI monoclonal antibodies. Results are expressed as percentage of fluorescent cells (background values, obtained by staining the cells with ann anti-TNF monoclonal antibody, are subtracted). For other details, see Materials and Methods..

Claims

1. A method for the productition of a soluble recombinant protein selected from human Tumor Necrosis Factor Binding Protein I (TTBP-I), biologically active precursors and analogs thereof, which comprises:
   i) transfecting eukaryotitic cells with an expression vector comprising a DNA molecule encoding the whole human type I TNFF receptor or a soluble domain thereof, and
   ii) culturing the transfeocted cells, whereby the desired protein is produced and secreted into the medium.

2. A method according to claaim 1 wherein the DNA molecule encoding the whole type I TNF receptor is the cDNA having the sequeence depicted in Figure 1D.

3. A method according to cblaim 2 wherein the cDNA is introduced into an expression vector and is cotransfected with a recoombinant vector containing the dihydrofolate reductase (DHFR) cDNA into DHFR-deficient chinese haamster ovary (CHO) cells.

4. A method according to claaim 3 wherein the cells are selected by growth in a nucleotide-free medium, individual clones are ampplified by growth in the presence of methotrexate and the soluble protein secreted into the mediumn is detected by reaction with monoclonal end polyclonal antibodies raised against TBP-I.

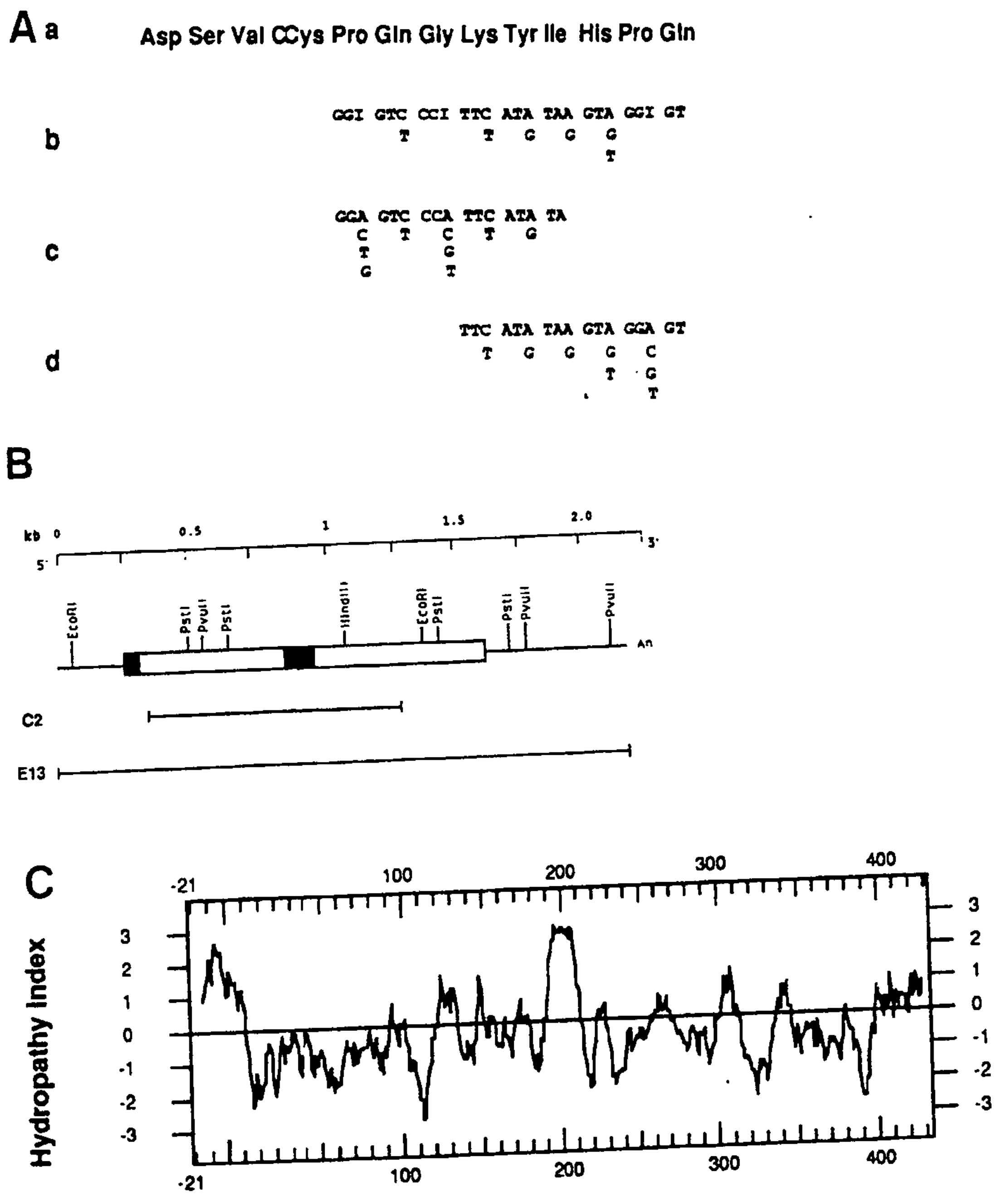
5. A method according to anny of claims 1 to 4 wherein the soluble protein secreted into the medium shows a retention time idenntical to that of TBP-I when analyzed by reversed phase HPLC.

6. A method according to anyy of claims 1 to 5 for the production of human TBP-I.

7. A method according to anyy of claims 1 to 5 for the production of a human TBP-I precursor or analog.

8. A soluble protein selected f from precursors and analogs of TBP-I which are secreted into the medium of eukaryotic cells transfecteod with a cDNA encoding the whole type I human TNF receptor or a soluble domain thereof.

9. A soluble protein as claimned in claim 8 secreted into the medium or CHO cells transfected with the cDNA molecule depicted irin Figure 1D.


A a
Asp Ser Val CCys Pro Gln Gly Lys Tyr Ile His Pro Gln
b
GGI GTC CCI TTC ATA TAA GTA GGI GT
T T G G G
T
c
GGA GTC CCA TTC ATA TA
C T C T G
T G
G T
d
TTC ATA TAA GTA GGA GT
T G G G C
T G
T
B
kb 0 0.5 1 1.5 2.0
5' 3'
EcoRI
PstI
PvuII
PstI
HindIII
EcoRI
PstI
PstI
PvuII
PvuII
An
C2
E13
C
Hydropathy Index
-21 100 200 300 400
3 2 1 0 -1 -2 -3

Fiigure 1 A-C

D

```
                                                                              1  CGGCCCAGTGATCTTGA     17
 18  ACCCCAAAGGCCAGAACTGGAGCCTCAGTCCAGAGAATTCTGAGAAAATTAAAGCAGAGAGGAGGGGAGAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAACCCTCAA     136
137  CTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCCCCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGGGGGAGTGAGGCCATAGCTGTCTGGC     255

     -21                                    -10                                 -1   +1
     Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu
256  ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG     345
     *
     10                                      20                                      30
     Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
346  GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC     435

     40                                      50                                      60
     Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr
436  AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC     525

     70                                      80                                      90
     Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
526  GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC     615

     100                                     110                                     120
     Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu
616  CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC     705

     130                                     140                                     150
     Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
706  AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC     795

     160                                     170                                     180
     Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
796  TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC     885

     190                                     200                                     210
     Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
886  ACA GTG CTG TTG CCC CTG GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG     975
```

Figure 1 D (part 1)

```
     220                                          230                                          240
     Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn
976  TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT AAG CCC CTG GCC CCA AAC  1065
     250                                          260                                          270
     Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr
1066 CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC  1155
     280                                          290                                          300
     Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala
1156 CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC  1245
     310                                          320                                          330
     Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp Thr Asp Asp Pro Ala Thr Leu Tyr
1246 TCC GAC CCC ATC CCC AAC CCC CTT CAG AAG TGG GAG GAC AGC GCC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAC  1335
     340                                          350                                          360
     Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu Ile Asp Arg Leu Glu Leu
1336 GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG ATC GAT CGG CTG GAG CTG  1425
     370                                          380                                          390
     Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala Thr Leu Glu Leu
1426 CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG CGG CGC ACG CCG CGG CGC GAG GCC ACG CTG GAG CTG  1515
     400                                          410                                          420
     Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro Pro Ala
1516 CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GCC GCC CTC CCG CCC GCG  1605
```

Figure 1 D (part 2)

```
     430
     Pro Ser Leu Leu Arg End
1606 CCC AGT CTT CTC AGA TGA GGCTGCGCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTCTGGAAAGGAGGGGTCCTGCAGGGGCAAGC 1718
                         *
1719 AGGAGCTAGCAGCCGCCTACTTGGTGCTAACCCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCCGACAGTCAGCGCTGTGCGCGGAGAGAGGTGCGCCGTGGGCTCAA 1837
1838 GAGCCTGAGTGGGTGGTTTGCGAGGATGAGGGACGCTATGCCTCATGCCCGTTTTGGGTGTCCTCACCAGCAAGGCTGCTCGGGGGCCCCTGGTTCGTCCCTGAGCCTTTTTCACAGTG 1956
1957 CATAAGCAGTTTTTTTTGTTTTTGTTTTGTTTTGTTTTGTTTTTAAATCAATCATGTTACACTAATAGAAACTTGGCACTCCTGTGCCCTCTGCCTGGACAAGCACATAGCAAGCTGAA 2075
2076 CTGTCCTAAGGCAGGGGCGAGCACGGAACAATGGGGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAAAAAAAAAA 2176
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Figure 1 D (part 3)

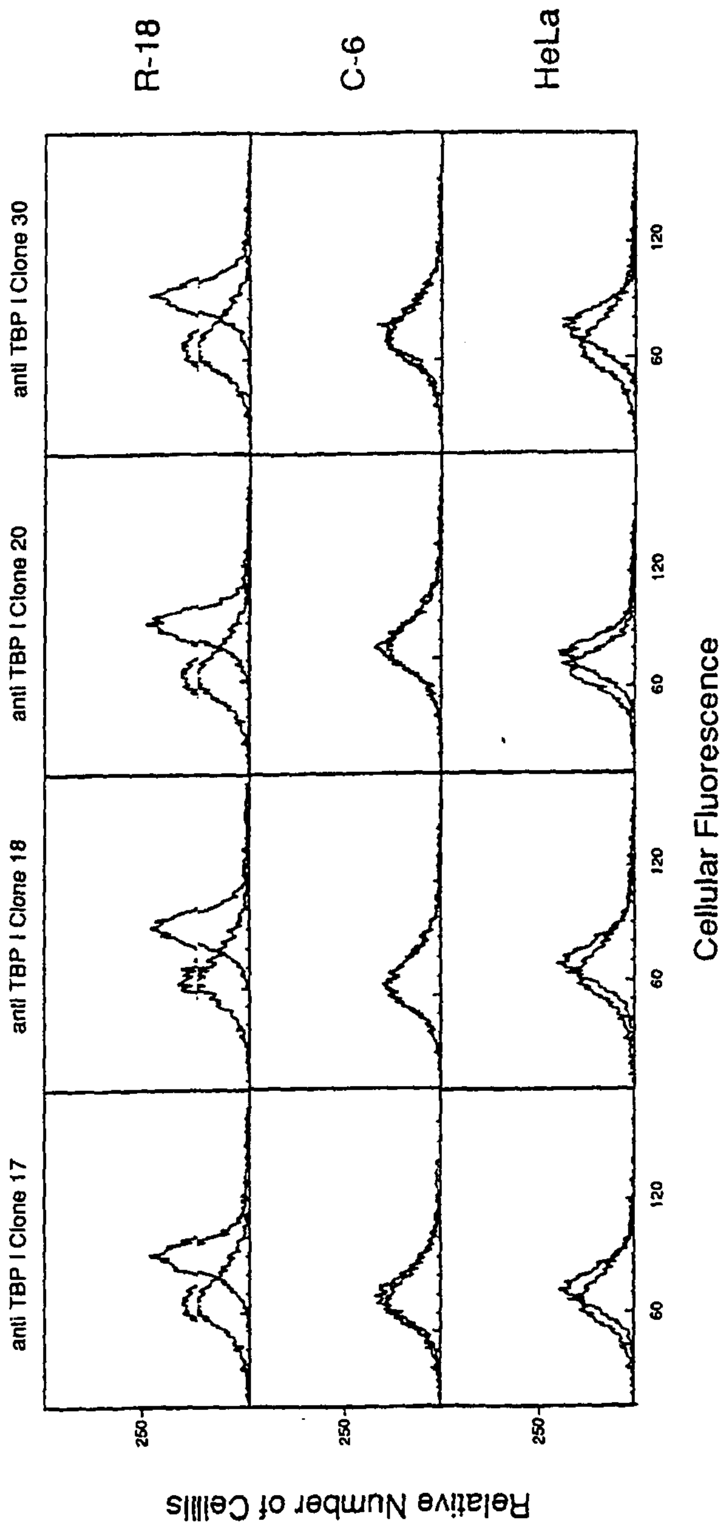

R-18
C-6
HeLa
anti TBP I Clone 17
anti TBP I Clone 18
anti TBP I Clone 20
anti TBP I Clone 30
250
250
250
60
120
Cellular Fluorescence
Relative Number of Cellls

Figure 2

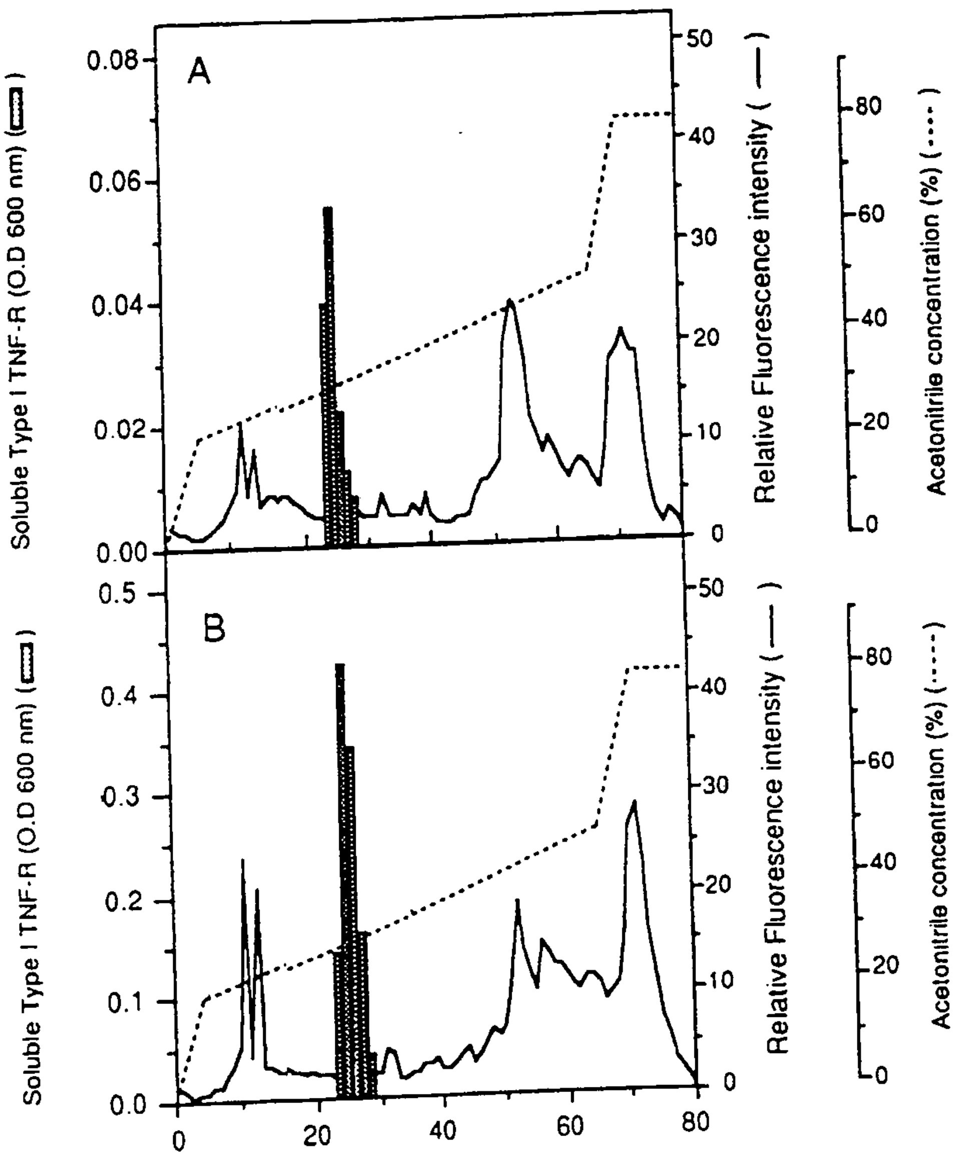
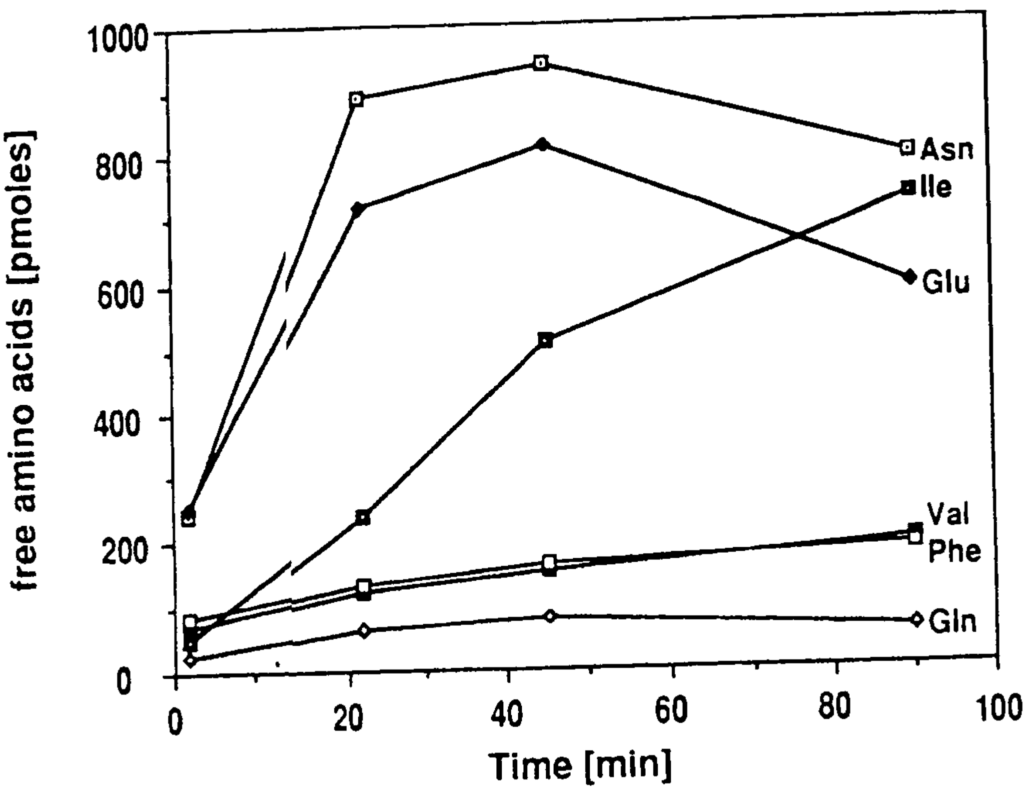

A
B
Soluble Type I TNF-R (O.D 600 nm)
Relative Fluorescence intensity
Acetonitrile concentration (%)
0.08
0.06
0.04
0.02
0.00
0.5
0.4
0.3
0.2
0.1
0.0
50
40
30
20
10
0
80
60
40
20
0
0
20
40
60
80

Figure 3


1000
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600
400
200
0
free amino acids [pmoles]
0
20
40
60
80
100
Time [min]
Asn
Ile
Glu
Val
Phe
Gln

Figure 4

CONSTRUCTION OF PLASMID pSV-TBP

TNF RECEPTOR cDNA

```
           -21                                                          180
           Met                                                          Asn
5'........ ATG GGC CTC TCC ACC GTG CCT . . . TGC CTA CCC CAG ATT GAG AAT ...      3'
3'. ..... .TAC CCG GAG AGG TGG CAC GGA .....ACG GAT GGG GTC TAA CTC TTA.......  . 5'
```

PCR Amplification

Phosphorilated 5' primer

```
5' CGG CCG ATG GGC CTC TCC ACC GTG CCT  . . .  . ..                          3'
3' ......... ...... . ....    .  .        . . . ACG GAT GGG GTC TAA CTC TTA TGA TAA 5'
```

Phosphorilated 3' primer

615 bp fragment

(coding for TBP I including its signal peptide)

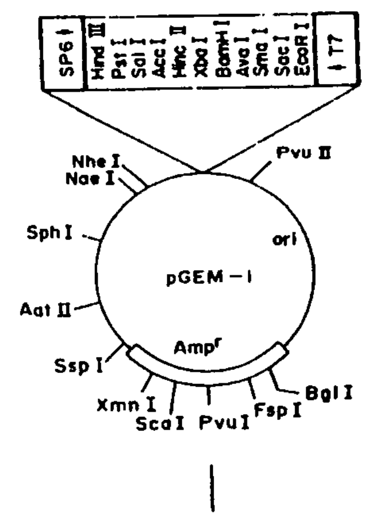

Figure 5 part 1)

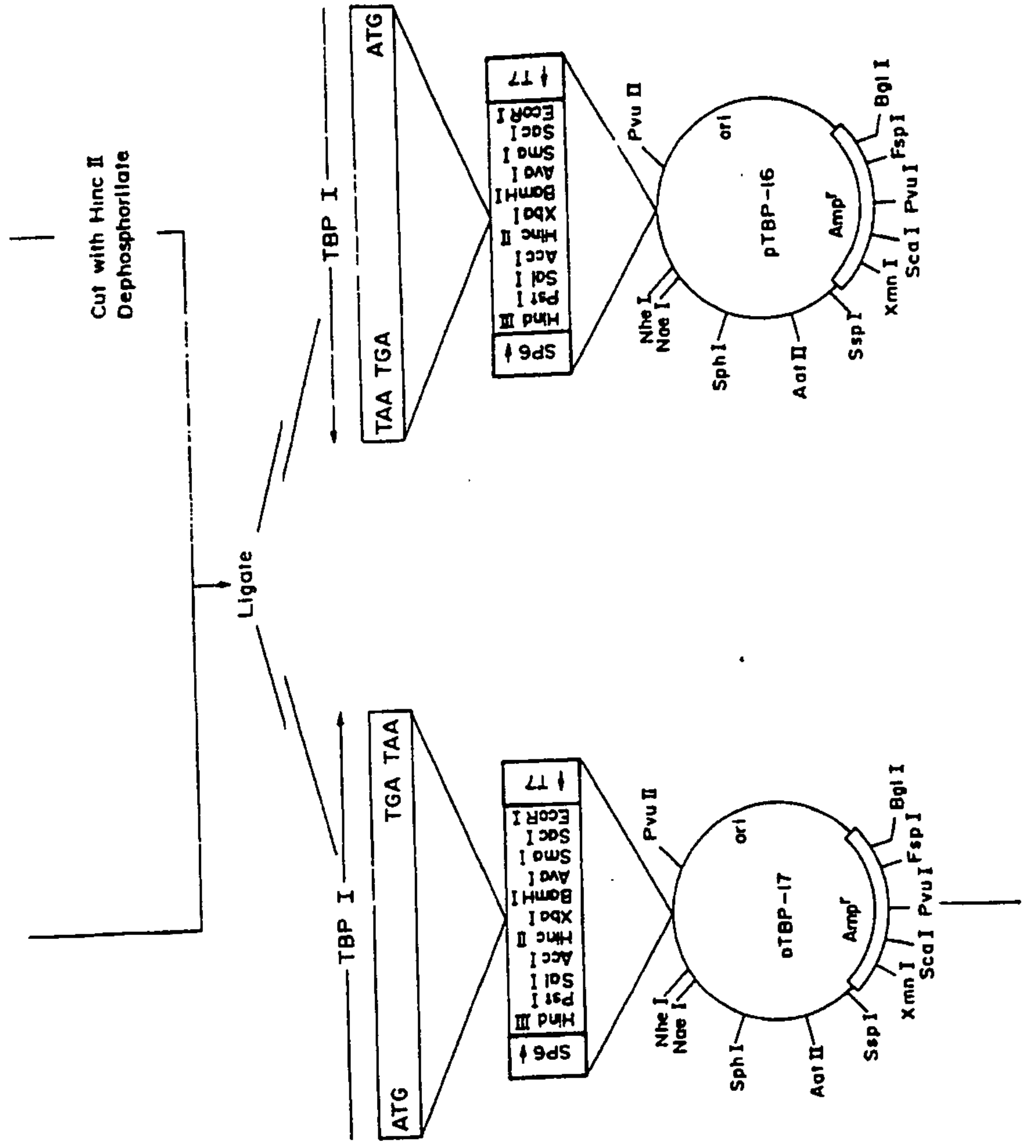

Figure 5 (part 2)

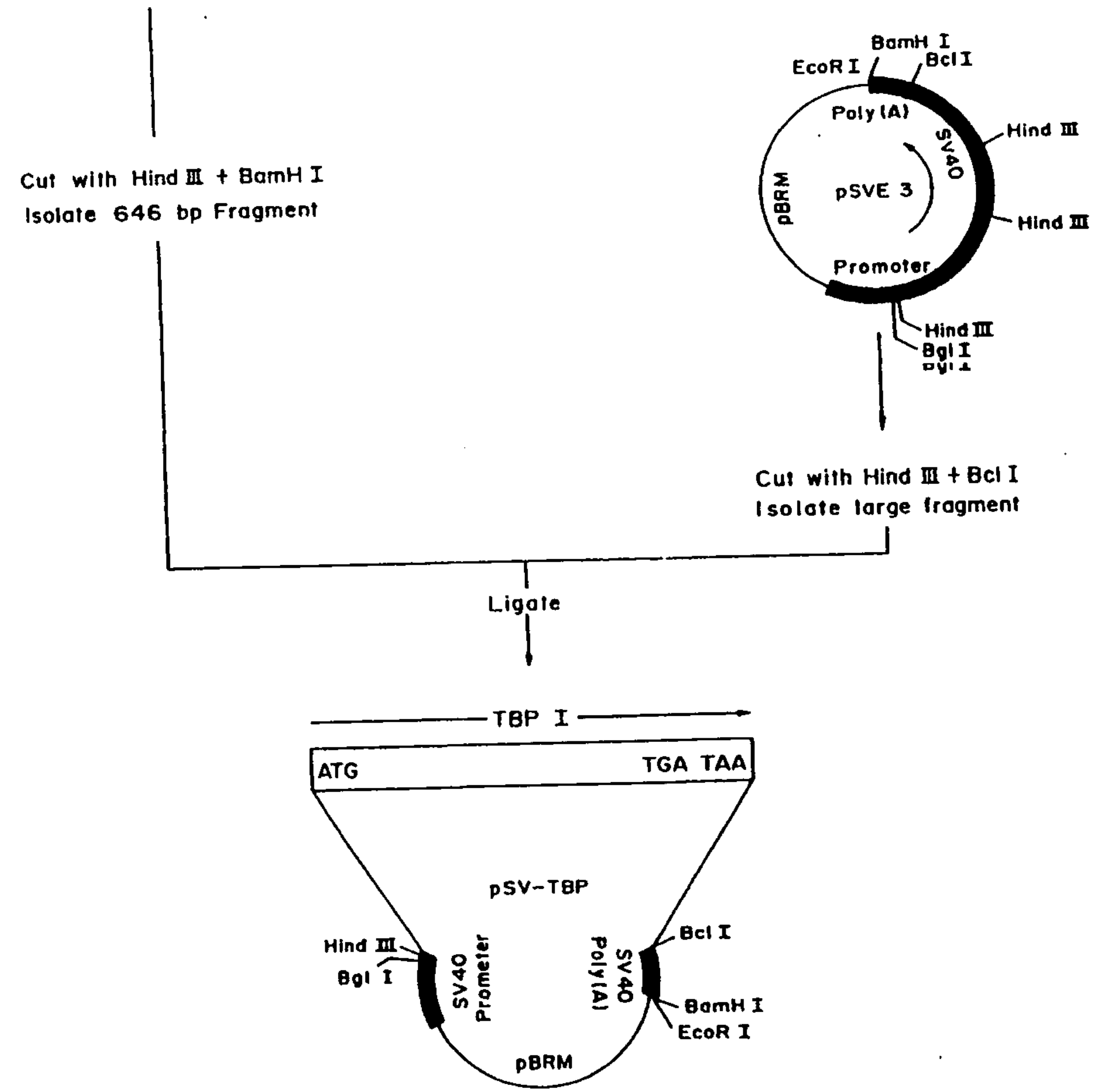

Figure 5 (part 3)

CONSTRUCTION OF PLASMID pCMV-TBP

TNF RECEPTOR cDNA

```
                 -21                                                        180
                 Met                                                        Asn
5'. . . . . . ATG GGC CTC TCC ACC GTG CCT    ..TGC CTA CCC CAG ATT GAG AAT. . . . . . 3'
3'.       ...  TAC CCG GAG AGG TGG CAC GGA. . . . ACG GAT GGG GTC TAA CTC TTA . . . . . . 5'
```

↓

PCR Amplification

↓

Phosphorilated 5' primer

```
5' CGG CCG ATG GGC CTC TCC ACC GTG CCT. . . . . . . . . . . . . . . . . . . . . . . 3'
3' . . . . . . . . . . . . . . . . . . . . . . . . . . . . ACG GAT GGG GTC TAA CTC TTA TGA TAA 5'
```

Phosphorilated 3' primer

↓

615 bp fragment
(coding for TBP I including its signal peptide)

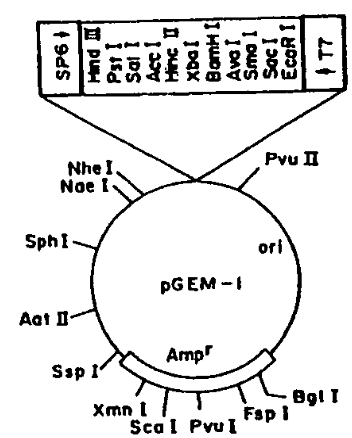

Figure 6 (part 1)

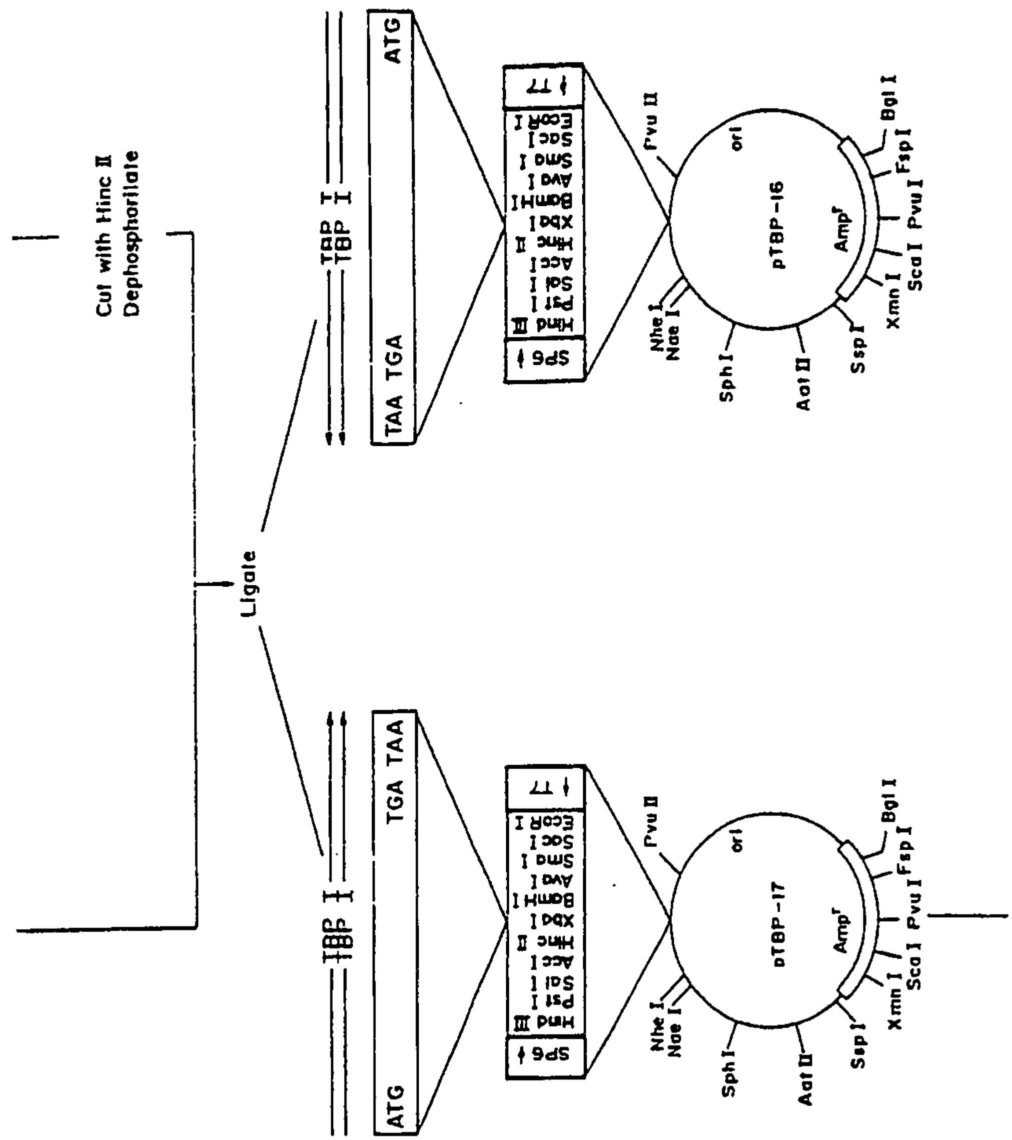

Figure 6 (part 2)

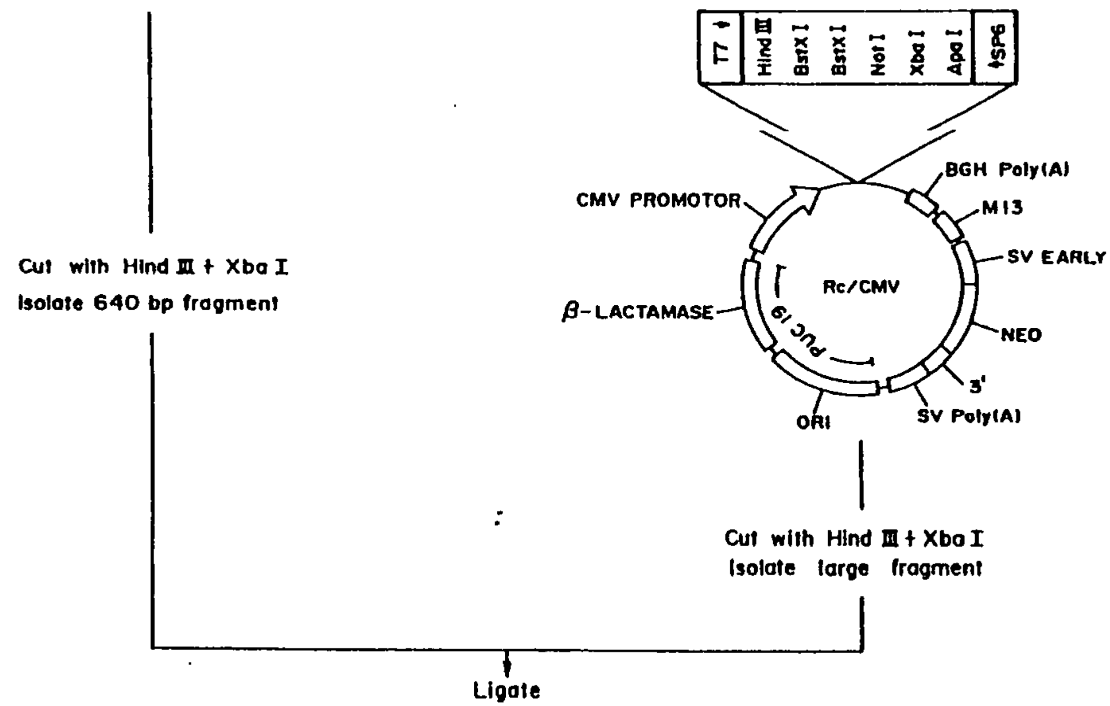

Figure 6 (part 3)

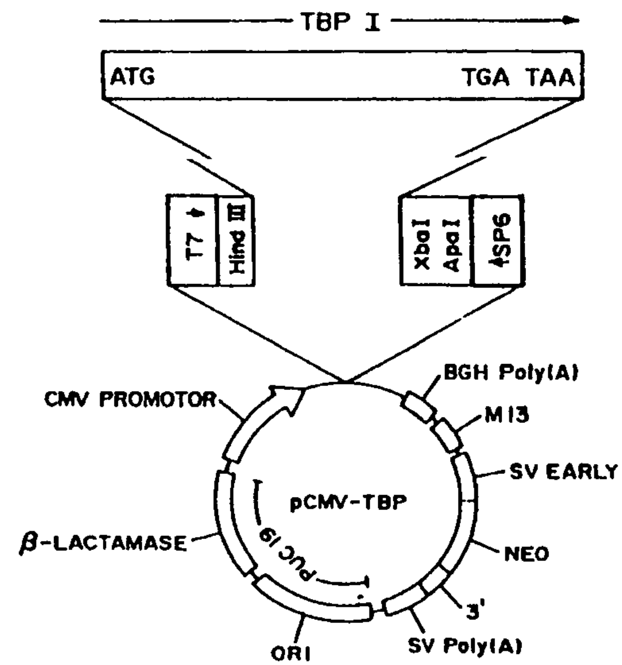

Figure 6 (part 4)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 12 4133

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,Y	EUROPEAN JOURNAL OF HAEMATOLOGY, vol. 42, no. 3, March 1989, pages 270-275; I. OLSSON et al.: "Isolation and characterization of a tumor necrosis factor binding protein from urine" * Whole document *	1-3,5-9	C 12 N 15/12 C 07 K 13/00 C 12 N 15/79
Y	THE EMBO JOURNAL, vol. 8, no. 9, September 1989, pages 2497-2502; C. BINKERT et al.: "Cloning, sequence analysis and expression of a cDNA encoding a novel insulin-like growth factor binding protein (IGFBP-2)" * Summary *	1-3,5-9	
P,X	CELL, vol. 61, 20th April 1990, pages 351-359, Cambridge, MA, US; H.R. LOETSCHER et al.: "Molecular cloning and expression of the human 55kd tumor necrosis factor receptor" * Summary; figure 2; Experimental procedures *	1,2,5-9	
P,X	PROC. NATL. ACAD. SCI. USA, vol. 87, 14th November 1990, pages 8781-8784, US; T. GATANAGA et al.: "Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients" * Whole document *	1,2,5	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 N C 07 K
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 10, 5th April 1990, pages 5708-5717, US; R.A. HELLER et al.: "Amplified expression of tumor necrosis factor receptor in cells transfected with Epstein-Barr virus shuttle vector cDNA librairies" * Abstract; page 5173, column 2, paragraph 2; page 5174, column 1, paragraph 1: "Discussion" *	1-3,6-9	

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	27 February 91	NAUCHE S.A.

CATEGORY OF CITED DOCUMENTS

X: particularly relevant if taken alone
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DOCUMENTS CONSIIDERED TO BE RELEVANT

Category	Citation of document witith indication, where appropriate, of releevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EUROPEAN JOURNAL OF HAEMATOLOGY, vol. 41, no. 5, November 1988, pages 4144-419; C. PEETRE et al.: "A tumor necrosis factor bindinng protein is present in biological fluids" * Whole document * - -- - - -	1-9	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has b been drawn up for all claims

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D: document cited in the application
L: document cited for other reasons

&: member of the same patent family, corresponding document